UNITED STATES PATENT APPLICATION

For:

DEVICE FOR MONITORING ENZYMES

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BACKGROUND OF THE INVENTION

1. Field of the Invention

This application is a continuation-in-part of U.S. Serial No. 09/342,720 filed on June 29, 1999, which is a continuation-in-part of United States Serial Number 08/715,557, filed on September 18, 1996, which is a continuation-in-part of United States Serial Number 08/025,899, filed on March 3, 1993, which issued as U.S. Patent No. 5,567,598 on October 22, 1996, and which is continuation of United States Serial Number 07/687,359, filed on April 18, 1991.

2. Description of Related Art

Our environment contains a multitude of microorganisms with which we are continuously interacting. These interactions can be beneficial, i.e., fermentations to produce wine, vinegar or antibiotics; neutral; or even harmful, as in the case of infectious diseases. The ubiquitous presence of these microorganisms, thus, creates a continuing need for the detection, identification and study of the presence and metabolic activity of such microorganisms.

While the science of microbiology has changed significantly in the last 25 years, many procedures for the detection, identification and analysis of the behavior of microorganisms are still time consuming. For example, in the area of antimicrobic

susceptibility testing nearly half of all testing in hospitals in the United States still use the Bauer-Kirby Disc Method. This method uses the presence or absence of visible growth of the microorganisms to indicate the efficacy of an antimicrobic compound, and generally requires an 18 to 24 hour incubation period to allow for microorganism growth before a result can be obtained. A decrease in the time required to obtain such antimicrobic susceptibility information is needed.

Another popular method for antimicrobic susceptibility testing is the broth micro-dilution method, such as the Sceptor® System for identification and antimicrobic susceptibility testing or organisms (Becton Dickinson Diagnostic Instrumentation Systems, Sparks, MD). The system uses a disposable plastic panel having a plurality of low volume cupulas (ca. 0.4 ml per cupula), each containing a different test compound or a different concentration of a test compound dried on the cupula surface. The organism to be tested is suspended in the desired testing medium, and aliquots are delivered to the individual cupulas of the test panel. The reagent dried on the panel dissolves in the sample, and the system is then incubated overnight (18 to 24 hrs.) to allow sufficient time for the organisms to interact with the reagent and for visible growth to appear. The panel is subsequently examined visually for the presence or absence of growth, thereby obtaining information on the susceptibility of the organism undergoing testing. Additional wells aid in identifying the organism. However, this test method suffers from the drawback of also requiring a long incubation period.

One approach to the reduction of the incubation time is to monitor metabolic activity of the microorganisms, rather than growth of colonies. Many approaches have been reported in the attempt to rapidly and accurately monitor such metabolic activity.

For example, apparatus utilizing light scattering optical means have been used to determine susceptibility by probing the change in size or number of

microorganisms in the presence of various antimicrobic compounds. Commercial instruments utilizing these principles are exemplified by the Vitec System (BioMerieux Corp.). This system claims to yield information on antimicrobic susceptibility of microorganisms within 6 hours for many organism and drug combinations. Other combinations can require as long as 18 hours before the antimicrobic susceptibility of the organism can be determined by this machine.

Additionally, modifications of the Bauer-Kirby procedure have been developed which allow certain samples to be read in four to six hours. However, such a system is "destructive" in nature, requiring the spraying of a developing solution of a color forming dye onto the test plate. Re-incubation and reading at a later time is, thus, not possible and if the rapid technique fails, the experiment cannot be continued for a standard evaluation at a later time.

A bioluminescent method based on the quantity of ATP present in multiplying organisms has been described as yielding results of antimicrobic susceptibility testing in four and half hours for certain compositions (Wheat et al.). However, the procedure tends to be cumbersome and broad applicability has not been shown.

Other approaches have involved monitoring of microbial oxygen consumption by the measurement of pH and/or hemoglobin color change, or by the use of dyes such as triphenyltetrazolium chloride and resazurin, that change color in response to the total redox potential of the liquid test medium.

The monitoring of the consumption of dissolved oxygen by microorganisms, as a marker of their metabolism, has been studied for many years. For example, C.E. Clifton monitored the oxygen consumption of microorganisms over a period of several days using a Warburg flask in 1937. This method measured the change in oxygen concentration in a slow and cumbersome manner.

The "Clark" electrode, a newer electrochemical device, is also commonly used to measure dissolved oxygen. Unfortunately, the Clark electrode consumes oxygen during use (thereby reducing the oxygen available to the microorganisms) and the "standard" size electrode is typically used only to measure volumes of 100 mls or greater to prevent the electrode from interfering with the measurements.

A "miniature" Clark electrode has been described, but this electrode is a complicated multi-component part which must, also, be in contact with the solution to be measured. While an oxygen permeable membrane can be used to prevent the electrode components of the device from interacting with the constituents of the test solution, the oxygen must still equilibrate between the test solution and the measurement system and is consumed once it passes the membrane.

Optical systems which can yield oxygen concentration data, have been developed to overcome the shortcomings of the Clark electrode systems. The main advantage of such optical methods is that the instrumentation required to determine quantitative value does not itself make physical contact with the test solution. Optical techniques allowing both colorimetric and fluorometric analyses for oxygen to be carried out rapidly and reproducibly are known, and costs for such analyses are often quite low. For example, several luminescent techniques for the determination of oxygen have been described which are based on the ability of oxygen to quench the fluorescence or phosphorescence emissions of a variety of compounds. However, such methods have not been adapted to microbial monitoring or prokaryotic or eukaryotic cell monitoring.

Other systems have been described that provide information on the presence, identity and antimicrobic susceptibility of microorganisms in a period of eight hours or less. Wilkins and Stones in U.S. Patent No. 4,200,493 disclose a system that uses electrodes and a high impedance potentiometer to determine the presence of microorganisms. In U.S. 3,907,646 Wilkins et al. disclose an analytical method

which utilizes the pressure changes in the headspace over a flask associated with microbial growth for the detection and surveillance of the organisms. U.S. 4,220,715 to Ahnell, discloses a system wherein the head space gas above a test sample is passed through an external oxygen detector for determination of the presence of microorganisms. Ahnell, in U.S. Pat. No. 4,152,213, discloses a system for analysis by monitoring the vacuum produced by growing organisms in a closed head space above a test sample. U.S. 4,116,775 to Charles et al. is an example of the use of optical means based on the increase in turbidity or optical density of a growing microbial culture for the detection and monitoring of bacterial growth. A combined electro-optical measurement of birefringence of a test solution containing microorganisms is described in EPO 0092958 (Lowe and Meltzer).

The increased incidence of tuberculosis and the recent emergence of Multiple Drug Resistant (MDR) strains threatens the ability to control this disease. Therefore, when a strain is resistant to two or more drugs, such as rifampin and isoniazid, the course of treatment increases from 6 months to 24 months, and the cure rate decreases from almost 100% to less than 60%.

Mycobacterium tuberculosis (TB) is a slow growing species. Generally, at least three to five weeks of growth on solid or liquid media are required to produce enough cell mass for identification and susceptibility testing. The most commonly used susceptibility method for TB is the Modified Proportion Method (NCCLS M24-T). This method requires an additional three to four weeks of growth before the results are available. The total elapsed time for a find report is typically two months and may be as much as three months.

The BACTEC 460 instrument (Becton, Dickinson and Company, Franklin Lakes, NJ) can reduce these times considerably. The BACTEC method detects the presence of mycobacteria by their production of radioactive CO_2. The BACTEC

system can also detect resistant organisms by their continuing production of radioactive CO_2 in the presence of antimycobacterial drugs.

It becomes apparent that a wide variety of methods have been applied to the detection and the antibiotic susceptibility testing of microorganisms. Many of these methods can only yield useful data when monitored by instruments dedicated to this task. Thus there exists a need for a system which can allow determinations of the presence and behavior of microorganisms without the requirement of dedicated instrumentation. Further there exists a need for a system that will allow the determination of the effect of a compound such as an antibiotic on a sample of microorganisms in a short time that does not significantly alter the behavior of the microorganisms.

There also currently exists a need for improved methods of measuring eukaryotic and/or prokaryotic cell growth and viability, such as, for example, in the areas of drug discovery and development. An important application for these methods is in testing and quantifying the effects of therapeutic drugs, drug candidates, toxins and chemicals on the growth of cell lines (i.e, cytotoxicity assays). As an example, potential chemotherapeutic drug candidates are frequently tested at a number of concentrations to determine their potency for inhibiting the growth of selected mammalian tumor cell lines.

The most commonly used reagent for eukaryotic (i.e., mammalian) cell cytotoxicity assays is MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) ["Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays", T. Mossmann, J. Immunol. Methods (1983), vol. 65, 55-63]. This tetrazolium salt is reduced within the mitochondria of metabolically active cells to form a colored precipitate (formazan dye). For cytotoxicty measurements, the cells are typically grown in a microwell trays containing various concentrations of drug. MTT is added and incubated with cells for

1-4 hours, the cells are lysed, the formazan dye is resolubilized by thorough mixing and a dose-response curve is obtained from endpoint absorbance measurements. Among disadvantages of this method are the multiple reagent additions which are required. MTT is also susceptible to interferences from some drugs with reducing groups and from precipitation of some drugs, especially those adsorbing light in the visible region. The test itself is non-reversible and further time point readings of the same cell cultures cannot be performed without setting up a separate assay to be used for each time point.

Another redox indicator suggested for cytotoxicity assays is resazurin which is reduced to resorufin in the presence of growing cells. Resazurin is subject to autoreduction in some media which can cause false positive signals. An improved formulation of resazurin with a redox stabilizing buffer known as "Alamar Blue" has been introduced to solve this autoreduction problem in U.S. Patent No. 5,501,959. This formulation, however, still requires the addition of dye and buffer to the cells and is essentially a non-reversible reduction.

Another method for determining cell viability is to measure uptake of radiolabeled nucleotides such as tritiated thymidine. This test is very sensitive but it is relatively expensive, time-consuming, and requires multiple steps. It also requires the handling and disposal of radioisotopic waste. This type of assay cannot readily be automated or adapted to formats for rapid drug screening purposes.

Furthermore, one of the major bottlenecks within the drug discovery process of the pharmaceutical industry is in the area of drug metabolism. The development of *in vitro* assays systems that are rapid, user friendly and automatible would do much to alleviate this backlog. Drug metabolic reactions can be generally divided into two areas: Phase 1 reactions which involves chemical alteration of the drug (e.g. oxidation, reduction or hydrolysis); and Phase 2 reactions, in which the drug moiety is conjugated (e.g. glucuronidation, sulphation, etc.)

The majority of xenobiotics are metabolized through the CYP450 system, which is also involved in the oxidative metabolism of many endogenous compounds. The CYP450s catalyze an oxidative reaction that is characterized by the oxidation of a substrate (R) using atmospheric oxygen (O_2). The reaction requires a reduced cofactor such as NADPH + H and can be expressed as the following reaction:

$$NADPH + H+ + RH + O_2 \rightarrow NADP+ + ROH + H_20. \quad (1)$$

However, the P450 family of enzymes is known to be highly promiscuous, allowing each P450 isozyme to metabolize a number of different substrates. Because of their relative non-specificity, the P450s also catalyze non-productive or "uncoupled" reactions that produce H_2O and H_2O_2, in addition to the productive pathway that catalyzes the oxidation of xenobiotics. These reactions are shown in equations (2) and (3). ["On the stoichiometry of the oxidase and monooxygenase reactions catalyzed by liver microsomal cytochrome P-450", L.D. Gorsky, D.R. Koop, M.J. Coon, J. Biol. Chem. (1984), vol. 259, 6812-6817].

$$NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O_2 \quad (2)$$

$$2\,NADPH + 2\,H^+ + O_2 \rightarrow 2\,NADP^+ + 2H_2O \quad (3)$$

Knowledge of the rates of oxygen consumption, peroxide formation, and NADPH consumption allows for an unambiguous determination of the absolute rates of each of these 3 reactions. The system of the present invention allows for substantially simultaneous measurement of these three reaction rates by multiplexing three different fluorescence detection methodologies.

SUMMARY OF THE INVENTION

It is therefore an object of this invention to provide an improved means to detect the presence of, and to evaluate the metabolic activity of, one or more

enzymes present in a liquid or semi-solid media. It is further an object of this invention to provide a microbial monitoring device or system which can be simply read and visually interpreted, and which permits results to be obtained in a shorter time period than previously attainable, nominally 6 hours or less. Additionally, it is an object of the invention to provide a means for detecting and/or monitoring the activity of enzymes without the use of dedicated instrumentation.

The above and related objects are realized by the processes of the instant invention. These processes utilize a luminescence detection system, and more particularly, a fluorescence detection system wherein the fluorescing sensor compound is one which exhibits a quantifiable degree of quenching when exposed to oxygen. In one embodiment, the sensor compound may be brought into contact with the test sample (either directly or separated by an oxygen permeable membrane) and the fluorescence is measured or observed visually with appropriate aids. In another embodiment, an increase in fluorescence is indicative of oxidative reactions catalyzed by enzymes, which utilize (and thereby reduce) the oxygen in the sample.

The sensor need not be in direct contact with the test sample. The only requirement is that the test sample and sensor are in a container substantially isolated from atmospheric oxygen so that the sensor can react to the presence/absence of oxygen in the container.

The system can, thus, be used to detect a variety of oxidative reactions catalyzed by enzymes.

Specifically, the system of the present invention can be used as a rapid *in vitro* screening assay for assessing the rate of metabolic elimination of compounds that are toxic or are not effectively metabolized.

This rapid screening would reduce the number of compounds requiring analysis by liquid chromatography / mass spectrometry (LC/MS) and thus alleviate a bottleneck that currently exists in the pharmaceutical industry.

In the current state of the art, the most common method for assessing the rate at which a chemical entity is metabolized by the enzymes in the liver (the rate of "clearance") is to perform an *in vitro* assay (in a microplate) wherein an array of chemical entities is exposed to one or more preparations of CYP450 enzymes. After a specified period of time – typically 10 - 60 minutes – the contents of each and every well of the microplate must then be analyzed to see what happened. LC/MS is an enhanced chromatographic process which not only separates the components in the sample (the LC aspect), but also reveals the molecular weight corresponding to each peak (the MS aspect). LC/MS is thus a very powerful technique; the information so obtained allows determination of not only whether the compound has been metabolized, but also gives insight into what the metabolites (products of metabolism) are.

The problem is that LC/MS is an extremely expensive way to accomplish the primary objective of a screening assay, which is simply to eliminate from contention for further development those compounds which fail to exhibit a rate of clearance that falls within the desired window. LC/MS is expensive, both because the hardware costs hundreds of thousands of dollars, and because it is an inherently serial process, meaning that once the one-hour assay is completed, there is then a long queue of samples to be analyzed one after another. Following this, the data results of the LC/MS must be processed in order to ultimately assess which compounds were metabolized at what rates, meaning there is a very long wait before the researchers have the answer they are attempting to find. Because of the delay it imposes between performing the assay and getting the results, and because of its inherent serial processing nature, the LC/MS step in the process has become a bottleneck.

What the present invention offers is the ability to ascertain, without having to use LC/MS, the relative rates of metabolic clearance via the relative rates of oxygen consumption, peroxide formation, and NADPH consumption, simply by reading the wells in a plate reader, an inherently parallel analytical process. Hence, the LC/MS bottleneck is eliminated, and researchers may ascertain rank order information concerning metabolic stability in a rapid and high-throughput manner.

The system of the present invention would allow for the multiplexing of different types of fluorescent labels or for the multiplexing of different assays (e.g., cellular metabolism and toxicity).

The system of the present invention shall facilitate real time kinetic profiling of multiple samples in one plate.

This system may also be used in cytoxicity assays for the effects of drugs, toxins, or chemicals on such reactions.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 graphically depicts intensity of fluorescence as a function of time for indicators in contact with broth containing organisms and broth containing no organisms.

Figure 2 graphically depicts the intensity of fluorescence as a function of time for indicators in contact with broth inoculated with different concentrations of microorganisms.

Figure 3 graphically depicts the intensity of fluorescence as a function of time for indicators in contact with broth inoculated with the same number of organisms but containing different concentrations of phenol.

Figure 4 graphically depicts the intensity of fluorescence as a function of time for indicators in contact with broth inoculated with the same number of organisms but containing different amounts of copper sulfate.

Figure 5A graphically depicts the fluorescence, as a function of indicators in contact with broth inoculated with the same concentration of microorganisms but different concentrations of cefuroxime. Some wells were covered with mineral oil to prevent oxygen from diffusing into the wells. The fluorescence is given as a percent of growth control.

Figure 5B graphically depicts the fluorescence as a percent of the growth control in wells that are overlaid with oil or left open and measured at several different times.

Figure 6 graphically depicts the intensity of fluorescence of indicators in blood culture bottles when measured continuously over 16 hours. The arrows indicate the times when samples were removed in order to quantify the concentration of organisms present.

Figure 7 depicts the data collected in the BACTEC® instrument indicating the change in fluorescence intensity indicative of the growth of *P. aeruginosa*.

Figure 8 depicts the data collected in the BACTEC® instrument indicating the change in fluorescence intensity indicative of the growth of *M. fortuitum*.

Figure 9 depicts the data collected in the BACTEC® instrument indicating the change in fluorescence intensity indicative of the growth of *E. coli*.

Figure 10 graphically depicts intensity of fluorescence by plotting fluorescence signal vs. cell number for HL60 cells growing in oxygen sensor plates, wherein the cell number was determined by averaged hemacytometer readings.

Figure 11 graphically depicts intensity of fluorescence by plotting fluorescence signal vs. the initial cell number for U937 cells grown in oxygen sensor plates.

Figure 12 graphically depicts intensity of fluorescence by plotting fluorescence vs. concentration of vinblastine in a cytotoxicity assay with HL60 cells at selected time points in an oxygen sensor plate.

Figure 13 graphically depicts absorbance, by plotting absorbance vs. concentration of vinblastine in a cytotoxicity assay using MTT with HL60 cells.

Figure 14 graphically depicts intensity of fluorescence by plotting fluorescence vs. concentration of methotrexate in a cytotoxicity assay with HL60 cells at selected time points in an oxygen sensor plate.

Figure 15 graphically depicts intensity of fluorescence by plotting fluorescence vs. concentration of sodium azide in a cytotoxicity assay with HL60 cells at selected time points in an oxygen sensor plate.

Figure 16 graphically depicts intensity of fluorescence by plotting fluorescence vs. concentration of SDS in a cytotoxicity assay with HL60 cells at selected time points in an oxygen sensor plate.

Figure 17 graphically depicts intensity of fluorescence by plotting fluorescence vs. time for oxygen sensor plates in which MCD-1 cells were grown on the indicated amounts of MATRIGEL®.

Figures 18A, 18B and 18C graphically depict intensity of fluorescence by plotting fluorescence vs. time for oxygen sensor plates in which MCD-1, SK-N-SH, and NIH3T3 cell lines were grown on MATRIGEL®.

Figure 19A graphically depicts relative changes in the intensity of fluorescence over time for HL60 cells grown in a 24-well plate (i) with the sensor on the bottom of the well (unmodified RuSi); (ii) with the sensor on the bottom of an insert. The third data set is for a control of media only with no cells. Figure 19B shows the same experiments as in Figure 19A, but using adherent cell line MDCK instead of HL60 cells.

Figure 20 graphically depicts the normalized fluorescence intensity vs. the number of HL60 cells, grown in 96 well oxygen sensor plates and in 384 well sensor plates.

Figure 21 graphically depicts relative changes in the intensity of fluorescence by plotting fluorescence vs. time for SF-9 insect cells grown at the indicated concentrations in a 96 well oxygen sensor plate.

Figure 22 graphically depicts relative changes in the intensity of fluorescence by plotting fluorescence vs. time for yeast cells grown at the indicated concentrations in a 96 well oxygen sensor plate.

Figure 23 graphically depicts the relationship between the initial concentration of yeast cells and the time required to reach 120% of the initial fluorescence signal for yeast growing in media with the four indicated glucose concentrations.

Figure 24 graphically depicts the relationship between normalized fluorescence and time for reactions between glucose oxidase at 5 U/mL and various concentrations of glucose.

Figure 25 graphically depicts the relationship between the equilibrium normalized fluorescence and the concentrations of glucose oxidase and glucose used to generate the signal.

Figure 26 graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing various concentrations of CYP450 2C9 in the absence of substrate.

Figure 27 graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing a buffer-only negative control, CYP-2C9 alone, and CYP-2C9 plus 200 uM of a known substrate, diclofenac.

Figure 28 graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing 10 pmol of CYP- 2C9 and either zero or 200 uM of diclofenac. The black symbols represent wells in which the wells were overlaid with 50 uL of mineral oil, whereas white symbols represent corresponding wells for which mineral oil was not added.

Figure 29 graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing 10 pmol of CYP- 2C9 and (o) buffer alone, (●) CYP-2C9 alone, (□) CYP-2C9 plus 500 uM diclofenac, (▣)CYP-2C9 plus 500 uM diclofenac plus 25 uM sulphaphenazole, (■)CYP-2C9 plus 500 uM diclofenac plus 250 uM sulphaphenazole.

Figure 30 graphically depicts the relationship between the normalized oxygen-sensitive fluorescence and time for wells containing 10 pmol of CYP 2C9 and either 0, 100, or 500 uM ibuprofen.

Figure 31 graphically depicts the relationship between normalized peroxide concentration and ibuprofen concentration for wells containing 10 pmol of CYP -2C9 and either 0, 100, or 500 uM ibuprofen.

Figure 32a graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing 10 pmol of CYP 2D6 and either 0, 10, or 50 uM propranolol.

Figure 32b graphically depicts the relationship between NADPH concentration and time for wells containing 10 pmol of CYP 2D6 and either 0, 10, or 50 uM propranolol.

Figure 33a graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing 10 pmol of CYP 2D6 and either 100 uM propranolol or 100 uM dextromethorphan.

Figure 33b graphically depicts the relationship NADPH concentration and time for wells containing 10 pmol of CYP 2D6 and either 100 uM propranolol or 100 uM dextromethorphan.

Figure 34 graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing various numbers of hepatocyte Liverbeads.

Figure 35 graphically depicts the relationship between normalized oxygen-sensitive fluorescence and time for wells containing 45 Liverbeads and 200 uM diclofenac.

DETAILED DESCRIPTION OF THE INVENTION

The process of this invention presents a quick, easy, and unambiguous method for the measurement and/or detection of oxidative reactions catalyzed by one or more enzymes by measurement or visual observation of luminescence. The term luminescence is intended to include fluorescence and phosphorescence, as well as time-resolved fluorescence and fluorescence lifetime. In a preferred embodiment the luminescent sensor compound can be a fluorescent sensor compound. In the process of the present invention, this compound is irradiated with light containing wavelengths which cause it to fluoresce, and the fluorescence is measured by any standard means, or evaluated visually.

The fluorescent compound must be one which exhibits a large quenching upon exposure to oxygen at concentration ordinarily found in the test liquids (generally 0.4%). While virtually any such compound can be used, preferred fluorescent compounds of this invention are tris-2,2'-bipyridyl ruthenium (II) salts, especially the chloride hexahydrate salt ($Ru(BiPy)_3Cl_2$), tris-4,7-diphenyl-1,10-phenanthroline ruthenium (II) salts, especially the chloride (salt $Ru(DPP)_3Cl_2$), and 9,10-diphenyl anthracene (DPA).

The fluorescent compound must be placed in chemical communication with the oxygen of the test sample to exhibit the quenching. This can be achieved by placing the compound directly in contact with the sample. However, in a preferred embodiment the compound and sample are separated from each other by the interposition of a membrane embedding material permeable to oxygen, and relatively impermeable to the other sample components, between them, thereby preventing the interaction of the sample and the compound. Neither the fluorescent compound nor the membrane in which the fluorescent compound is embedded need be in direct contact with the test sample, broth, or fluid (the compound and sample must be substantially isolated from atmospheric oxygen, thereby preventing any false reading due to the presence of atmospheric oxygen), but still permitting reaction of the

compound to the presence or absence of oxygen as a result of the ready diffusion of oxygen through the membrane.

The system can be allowed to interact unobserved for a predetermined amount of time after which the presence or absence of fluorescence is observed and compared to appropriate control samples, yielding results that are often obtained with a single such observation. A particular benefit of this system is that the measurement of fluorescence is non-destructive and if after a period of time (e.g. 4 hours) the results are non-conclusive, the system can be re-incubated and read again at a later time. In fact, systems may be read as frequently as the user's reading device will allow, thereby facilitating the generation of kinetic data. Further, while it is anticipated that the results will be compared with reagent controls, such is by no means necessary, and it is postulated that, by appropriate choice of fluorescent compounds, a skilled technician or technologist would be capable of independently determining whether the results indicate the absence or presence of a proliferating culture or other oxygen-consuming reaction simply by observing the shape of the kinetic fluorescence profile.

The detection of fluorescent intensity can be performed by any means ordinarily used for such measurements, e.g. a fluorometer. Alternatively, the fluorescent intensity can be observed visually and, optionally, compared with a reagent control (e.g. a system containing no enzymes or a system with no added test chemicals). Thus, the methods can be utilized to both provide a quantitative measurement of relative activity, using a fluorometer, or a more qualitative estimate of such activity, by visual inspection.

In a preferred embodiment of this invention, the fluorescent compound is chosen such that it will exhibit little or no fluorescence in the presence of oxygen. This obviates the need for a control, as the person performing the test would interpret any appreciable fluorescence (i.e. beyond that of any nominal background

fluorescence) as indicative of the presence of microbial activity. Such results can be obtained by a fluorometer or other measurement means, or preferably, visual inspection, and provide a quick, qualitative estimate of such activity. Preferred fluorescent compounds for this embodiment include $Ru(BiPy)_3Cl_2$ and $Ru(DPP)_3Cl_2$.

It has also been found that for systems where the compound or compound embedded membrane is in contact with the fluid, test sample, or broth, while the test can be run in systems isolated from atmospheric oxygen, accurate results can also be obtained when the system is left exposed to atmospheric oxygen. In fact, this is desirable when the organisms are to be incubated for periods of time exceeding 2 hours, as they would otherwise tend to consume all the dissolved oxygen in the system and subsequently generate a false reading. Thus, the system of this invention is quite versatile, and can be used in a wide array of conditions.

A further benefit of the instant invention is that a unitized apparatus can be constructed. Briefly, the apparatus comprises a sample containing reservoir, or more commonly a plurality of identical reservoirs adapted to contain a test sample and other such liquid and soluble components (e.g. nutrients, etc.) as may be required by the particular application. The reservoirs also provide a luminescent indicator element which monitors the oxygen levels of the solution. The indicator element of this invention uses a luminescent compound known to show a large quenching of its luminescent emission when exposed to oxygen.

In a preferred embodiment of this invention, the luminescent compound can be mixed and distributed throughout a plastic or rubber phase that is permeable to oxygen gas but relatively impermeable to water and non-gaseous solutes. Silicone rubber is a particularly useful material for this application. When a test solution containing, for example, microorganisms, is placed in such a sample reservoir, the metabolic activity of the organisms causes a reduction in the level of dissolved oxygen in the sample, and the sample will yield a higher luminescent signal upon

excitation. Sample liquids not containing microorganisms will not show a decrease in their oxygen levels and will only show low levels of luminescent due to high oxygen quenching of luminescence.

Alternatively, the oxygen sensitive fluorophore or luminescent compound can be in a microencapsulated form or in the form of granules of an oxygen permeable material. It is also anticipated that the fluorophore or luminescent compound can be contained within a separately manufactured component such as a bead, disc, or prongs, which can be separately introduced into the test solution. The use of prongs is particularly advantageous as such prongs can be attached to a lid or other device to permit easy manipulation. In a preferred embodiment, a plurality of prongs can be attached to a single membrane, or other cover and thereby be maintained in an appropriate orientation such that they can simultaneously be placed into the reservoirs of a base containing a plurality of sample reservoirs. By choice of appropriate materials, the prongs can be made impermeable to the indicator molecules and to microorganisms in the sample, but permeable to oxygen.

The fluorophore or luminescent compound can also be in a liquid phase separated from the solution being analyzed by a membrane that is impermeable to the indicator molecules and to microorganisms in the sample but which is permeable to oxygen. Additionally, less-sensitive sensors can be fabricated by using less O_2 permeable polymers or by using compounds with shorter excited-state lifetimes.

It is also considered that the luminescent sensor compound, which is an oxygen sensor, can be a phosphorescent compound such as platinum (II) and palladium (II) octaethyl porphyrin complexes immobilized in PMMA (polymethyl methacrylate); CAB (cellulose acetate brityrate); platinum (II) and palladium (II) octaethyl porphyrin ketone complexes immobilized in PVC (polyvinylchloride) and polystyrene.

Further, the methods of this invention can be used to test the susceptibility of organisms or enzymes to a compound, such as an antibiotic, which is capable of severely inhibiting the growth and/or the metabolic activity of the organisms or enzymes. The increase in luminescent signal normally caused by the organisms or enzymes will be suppressed in the presence of such compounds. The behavior of the luminescent signal from a reservoir will demonstrate the ability of the test component to negatively effect the normal oxygen consumption of the organism or enzyme added to the reservoir.

In addition, any of the embodiments discussed above may be utilized so that the sensor, luminescent compound, or the membrane in which it is embedded need not be in direct contact with the test sample, fluid, or broth in which the enzyme or oxidative reaction(s) catalyzed by enzymes may be present. In such case, the sensor, compound or membrane in which it is embedded need only be in the same container with the test sample, fluid or broth and that they be substantially isolated from atmospheric oxygen to function as an indicator of the presence or absence of enzymes and/or oxidative reactions catalyzed by enzymes.

It is also apparent that an assay method which is reversible, non-destructive to enzymes, requires no reagent additions, and poses no additional disposal requirements would be advantageous for cytotoxic drug screening, cellular quantitation, and viability testing.

The present invention describes a method for analyzing and quantifying oxidative reactions catalyzed by one or more enzymes based on their consumption of oxygen. Examples of such reactions and such enzymes include oxidative metabolic enzymes such as the CYP450 enzyme system, monoamine oxidase (MAO), and flavin monooxygenase (FMO). Other examples of oxygen-consuming or oxygen-producing reactions which may be monitored via the instant invention include the oxidation of glucose by glucose oxidase or the conversion of hydrogen peroxide

to water and oxygen by catalase. In a preferred embodiment, the oxidative reactions are performed by metabolic enzymes in liver cells (hepatocytes) or cells which have been modified to express metabolic enzymes. The oxidative reactions can be performed by enzyme systems (several enzymes which together comprise a subcellular system) or enzymes as described above. Examples of enzymes in the CYP450 enzyme system include cytochrome P450 and P450 reductase. These examples are not intended to restrict in any way the types of oxidative reactions and enzymes that can be analyzed by the present invention.

Furthermore, the sensor need not be in direct contact with the solution in order to analyze and quantify enzymes or oxidative reactions catalyzed by enzymes. The only requirement is that the solution and sensor are in a contained area substantially isolated from atmospheric oxygen so that the sensor can react to the presence or absence of oxygen in the contained area.

In a preferred embodiment, which is not intended to limit the present invention in any way, the use of such oxygen sensors in a microwell tray format for quantitation of metabolism of compounds by CYP450-containing enzyme preparations and for cytotoxicity assays is described. The microwell format enables reading with routine luminescence plate readers. This format offers ease of use in a non-destructive assay in which no additional reagents are required. This feature allows oxidative reactions catalyzed by enzymes to be repeatedly monitored since no dyes or indicators are added to or released into the cellular media. The oxidative reactions monitored in the wells may thus be removed and used for additional assays if desired. Additionally, because the monitoring of oxygen consumption requires no additional reagents, another assay may simultaneously be conducted within the same wells without fear of chemical interaction. Because this method is readily adapted to microwell tray formats such as 96 well and 384 well plates, the method is especially useful for high throughput screening of drugs, toxins and other chemicals to determine their cytotoxic activity.

Examples of drugs and toxins which can be utilized in the process of the present invention include gallium nitrate, procarbazine, fludarabine, vinblastine, streptozotocin, pentostatin, mitoxantrone, hydroxyurea, piperazinedione, MGBG, 5-azacytidine, bisantrene, cytarabin, colchicine, cladribin, amsacrine, 6-thioguanine, aclarubicin, cisplatin, 5-fluorourocil, bleomycin, mitomycin C, actinomycin D, methotrexate, mechlorethamine, melphalan, docetaxel, epirubicin, etoposide, vincristin, doxorubicin, teniposide, trimetrexate, topotecan, CPT 11, paclitaxel, gemcitabin, thymidine, acivicin, spirogermanium, cyclocytidine, zinostatin, flavone acctate, diglycoaldehyde, deazauridine, anguidine, PALA, aphidicolin, L-alanosine, maytansine, DQ-1, camptothecin, cremophor EL, homoharringtonine, sodium azide, DQ-2, and $HgCl_2$, but this is not intended to be limited to such drugs and toxins and can include any drug or toxin which can be utilized in the present invention.

Examples of chemicals, including components, compounds, amino acids, vitamins, salts, proteins and others, which can be utilized in the process of the present invention include magnesium chloride, glucose, D-galactose, L-valine, glutamine, phenylalanine, arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, tyrosine, valine, biotin, choline, folate, nicotinamide, pantothenate, pyridoxal, thiamine, riboflavin, sodium chloride, potassium chloride, NaH_2PO_4, $NaHCO_3$, calcium chloride, insulin, transferrin, and specific growth factors such as recombinant human epidermal growth factor, hydrocortisone, fibroblast growth factor, vascular endothelial growth factor, ascorbic acid (vitamin C), insulin-like growth factor and heparin, but this is not intended to be limited to such chemicals and can include any chemical which can be utilized in the present invention.

In a preferred embodiment, the oxygen sensor plates were prepared using 96 well microtiter plates following general methods described herein. These plates used

the fluorescent dye 1,7-diphenyl-1,10-phenanthroline ruthenium (II) chloride adsorbed to silica gel and embedded in a silicone matrix.

The sensor plates may be used to quantify, for example, the number of HL60 cells proliferating in media using a standard microplate fluorimeter. Results shown in Figure 10 for a preferred embodiment for quantifying cell number demonstrate that the absolute number of cells present during a growth assay correlates directly with the normalized fluorescent signal resulting from the cell's consumption of oxygen (the relative signal for each well at a given time point divided by the initial fluorescence of the well prior to the administration of the cell culture is referred to as "normalized fluorescence"). The absolute cell number was determined by hemacytometer at the time of each reading.

Another feature of these sensor plates is that they may be combined with additional biomaterials such as one or more extracellular matrices, such as, for example, collagen. Assays using the matrix MATRIGEL® with various cell lines are shown in Figures 17-18. These results indicate that coating the oxygen sensor plates with extracellular matrices does not hinder the ability to gain information on oxygen consumption.

Although the described plates use the luminescent dye 4,7-diphenyl-1,10-phenanthroline ruthenium (II) chloride, other luminescent dyes which exhibit significant oxygen quenching such as tris-2,2'-bipyridyl ruthenium chloride may also be used. A wide variety of silicone rubber polymers and other oxygen permeable polymers may be used to construct the sensors. Sensors may be constructed from sol-gel films ["Tailoring of Sol-Gel Films for Optical Sensing of Oxygen in Gas and Aqueous Phase", C. McDonaugh, B. D. MacCraith, and A. K. McElvoy, Anal. Chem. (1998), vol. 70, 45-50]. Alternatively, useful oxygen sensors can be constructed with the dye covalently immobilized to materials such as controlled pore glass ["Oxygen Sensing in Porous Glass with Covalently Bound Luminescent Ru(II) Complexes", M.

P. Xavier, et al, Anal. Chem. (1998), vol. 70, 5184-5189]. Other formats may include adding the sensor to unmodified plates in the form of beads or prongs [see U.S. Patent No. 5,567,598].

EXAMPLES

The following examples illustrate certain preferred embodiments of the instant invention but are not intended to be illustrative of all embodiments.

EXAMPLE 1. Preparation of an O_2-Sensitive Indicator Microtitration Tray

The fluorescent compound tris 4,7-diphenyl-1,10-phenanthroline ruthenium (II) chloride ($Ru(DPP)_3Cl_2$) was synthesized using the procedure of Watts and Crosby (J. Am.Chem. Soc. 93, 3184(1971)). A total of 3.6 mg of the compound was dissolved in 2.0 ml dimethyl sulfoxide (D-5879, Sigma Chemical, St. Louis, MO) and the resultant solution was then added slowly, with stirring, to 1300 ml silicone rubber forming solution (Water Based Emulsion #3-5024, Dow Corning, Midland, MI). A 35 microliter aliquot of the mixture was subsequently dispensed into each well of a 96 well, flat bottom, white microtiter tray (#011-010-7901, Dynatech, Chantilly, VA), and the system was subsequently cured overnight in a low humidity (less than 25% RH), 60°C incubator. After curing, the trays were washed by either soaking or by filling and emptying each well several times with each of the following reagents; a) absolute ethanol, b) 0.1 M phosphate buffer pH 7.2, c) hot distilled water (about 45°C) and d) ambient temperature distilled water.

Subsequently, 150 microliters of a Broth A, consisting of 35% Mueller Hinton II (BBL #124322, BD Microbiology Systems, Cockeysville MD), 15% Brucella (BDL #11088), and 50% distilled water, was dispensed into each well of the tray, and the tray was then placed in a glove box containing the desired concentration of oxygen, mixed with nitrogen to obtain a total pressure of 1 atm. The tray was kept in the glove box for at least 24 hours, after which it was covered with an adhesive backed mylar sheet and removed.

The fluorescent emissions of the fluorescent compound in the bottom of each well of the tray were then measured using a Perkin-Elmer LS-5B equipped with a microtiter reader attachment at the following instrument settings: 485nm excitation wavelength, 550nm cut-on filter in the emission window, 10nm excitation slit, and a 5nm emission slit. The results are presented in Table 1.

Table 1

Fluorescence of Tray Equilibrated with Various Oxygen Gas Levels

Tray	Average Reading	% Oxygen in Mixture (balance Nitrogen)
1	803	0.00
2	759	0.28
3	738	0.53
4	524	2.45
5	484	3.40
6	445	5.35
7	208	20.90

As shown, it can be observed that indicators in wells equilibrated in atmospheric air (Tray 7) displayed a much lower fluorescent signal than wells equilibrated with gas mixtures containing lower concentrations of oxygen (Trays 1-6). This indicates that the fluorescent emission of the fluorescent indicator compound embedded in the silicone rubber is related to oxygen concentration and that the system can be easily equilibrated with changing oxygen levels. The system allowed 96 sample wells (containing 0.1-0.3 ml sample) to be contained in a single unit that is easily manipulated.

EXAMPLE 2. Use of Indicator System to Measure Relative O_2 Concentration Produced by a Reducing Agent

The O_2 concentration in wells of an Indicator Microtiter tray produced as in Example 1 was varied by the addition of a strong reducing agent, sodium sulfite (which reduces O_2 content). A 150 microliter aliquot of the reducing agent (at

concentrations ranging from 0 to 1083 parts per million (ppm) sulfite ion in water) was pipetted into wells of the tray. Each well was allowed to react for 30 minutes, open to the atmosphere, and the fluorescence of the indicators measured in a Fluoroskan II Fluorometer (Flow Laboratories, McLean, VA), having an excitation bandpass filter at a wavelength of 460 nm and an emission cut-on filter at 570 nm. The results are presented in Table 2.

Table 2
Effect of Sodium Sulfite on Fluorescence

ppm sulfite ion	Fluorescence Intensity*
0	3090
65	3513
163	3545
325	4033
542	11571
1083	11863

* Mean of 4 wells

As shown, the wells containing the highest concentrations of reducing agent (and, consequently, the lowest O_2 concentration) have the highest fluorescence intensity, thus demonstrating the relationship between O_2 concentration and fluorescence.

EXAMPLE 3. Use of Indicator System to Determine the Presence of a Microorganism

A 0.5 McFarland suspension of E. coli (ATCC #25922), containing about 1.5 x 10^8 CFU/ml, was prepared using an A-Just nephelometer (Abbott Labs, Chicago, IL). The suspension was diluted to about 1 x 10^7 CFU/ml in Broth A (see Example 1). A 150 microliter aliquot of this suspension was placed into indicator tray wells prepared as in Example 1, and subsequently incubated at 37°C. At intervals, the fluorescence was measured in a Fluoroskan II fluorometer over the period of 1-3 ½ hours. An increased fluorescence signal was observed over time as shown in Figure 1. The fluorescence signal from wells containing no organisms showed very little change.

The wells containing organisms were significantly brighter when visually observed under a UV light source. Thus, it appears that the metabolic activity of the organisms in the wells caused the fluorescence signal to increase (presumably by decreasing the O_2 concentration).

EXAMPLE 4. Dependence of Fluorescence Change on Organism Concentration

A 0.5 McFarland suspension of E. coli (ATCC #29522) in sterile trypticase soy broth (TSB, BBL #11768) was made using an A-Just nephelometer (Abbott Labs, Chicago, IL). A series of E. coli suspensions ranging from 1 x 10^7 CFU/ml to about 10 CFR/ml were made by making serial dilutions. A 200 microliter aliquot of each suspension was placed into 8 wells of an indicator tray prepared as in Example 1. The tray was then incubated at 37°C and the fluorescence measured every 30 minutes in a Fluoroskan II fluorometer. The fluorescence of the 8 wells were averaged and corrected by subtracting the background fluorescence of a sterile TSB well. The change in fluorescence over time is shown in Figure 2.

As shown, a change in the starting concentration of the organism by a factor of 10 (one log unit) caused a delay of about 1 hour for the fluorescence in the well to exceed 2000 fluorescence units. It is postulated that this delay is due in part to the fact that the system is open to the atmosphere. Oxygen in the air can and does freely diffuse into the medium in an attempt to replace that consumed by the microorganisms. It is further postulated that only when the organisms are present in or have multiplied to sufficient numbers and are metabolically active enough to consume oxygen at a rate approximating or faster than the rate at which oxygen diffuses into the test solution, will the fluorescent signal generated by the indicator element in the bottom of the reservoir show an increase.

EXAMPLE 5. Preparation of an Indicator Microtitration Tray with an Alternat Fluorescent Indicating Molecule

A 96 well Microtiter tray was produced essentially as in Example 1, except that tris-(2,2' bipyridyl)-ruthenium (II) chloride hexahydrate (Aldrich Chemical Company, Milwaukee, WI) [$Ru(BiPy)_3Cl_2$] was substituted for $Ru(DPP)_3Cl_2$ in the silicone mixture. A second tray containing 9,10-diphenyl anthracene (DPA) was also prepared. All wells were charged with 150 ul of 1 x 107 CFU/ml E. coli (ATCC #25922) in broth. Table 3 lists the results at 0, 1, 2, 3, and 4 hours after addition of organisms.

Table 3

Fluorescence Counts for Devices with Different Fluorophores

Fluorescent Compound	Silicone	0 hr.	1 hr.	2 hr.	3 hr.	4 hr.
$Ru(DPP)_3Cl_2$ (Ex. 3)	A	2300	2315	2560	8329	9000
$Ru(BiPy)_3Cl_2$ (Ex. 5)	A	2866	--	3449	3951	4109
DPA (Ex. 5)	A	1300	--	1385	1456	1572
$Ru(DPP)_3Cl_2$ (Ex. 6)	B	--	995	4334	3775	3508

A = Dow-Corning 3-5024 water-based silicone
B = Wacker white SWS-960 + silicone

As shown, both fluorescent sensor compounds exhibited large increases with fluorescence over time, indicating their suitability for use in this system.

EXAMPLE 6. Preparation of an Indicator Microtitration Tray Using an Alternative Silicone

To demonstrate that the fluorophore can function when embedded in a different matrix, a 96 well Microtiter tray was produced essentially as in Example 1. In this experiment, 10 ul of white SWS-960 RTV silicone (Wacker Silicones, Adrian, MI) containing 10 milligrams of $Ru(DPP)_3Cl_2$ per liter was dispensed into each well of the tray and allowed to cure. No wash steps were performed on the resultant tray. The results are presented in Table 3. As in Example 1, wells containing 150 ul of 1 x 10^7 CFU/ml E. coli (ATCC #25922) in broth had a much greater fluorescent intensity after several hours at 37° Centigrade.

EXAMPLE 7. Eff ct of Toxic Substances on the Oxygen Consumption of Microorganisms

A suspension containing about 3 x 10^8 CFU/ml, of Pseudomonas aeruginosa (ATCC #10145) in Broth A was prepared using an A-Just nephelometer. A total of 150 ul of the suspension was placed in each well of the indicator trays prepared as in Example 1; these suspensions were then diluted with solutions of phenol or copper sulfate (which are deleterious to microbial growth) to a final concentration of 1.5 x 10^8 CFU/ml. The trays were incubated at 37°C and their fluorescence measured in a Fluoroskan II at 10 minute intervals. Figures 3 and 4 show the effect of phenol and copper sulfate on the response of the system.

As shown, at high levels of additives, growth was suppressed and the fluorescence did not increase with time. Wells containing phenol at 1 gram/liter or more, and copper sulfate at greater than 500 mg/liter, had no increase in fluorescence signal at times less than two hours, indicating absence of actively metabolizing organisms. Thus, measurement of oxygen consumption can be used to probe the metabolism of the organisms.

EXAMPLE 8. Effect of Antibiotics on E. coli

A 0.5 McFarland suspension of E. coli (ATCC #25922) in Broth A (see Example 1) was prepared using an A-Just nephelometer. The suspension was diluted to 1 x 10^7 CFU/ml in wells of an indicator tray prepared as in Example 1 containing the antibiotics ciprofloxacin, cefoxitin and cefuroxime at final concentrations of 0.5 to 8 ug/ml. The trays were incubated at 37°C for 4 hours and their fluorescence measured in a Fluoroskan II fluorometer. The results are presented in Table 4.

Table 4

Fluorescence from an Indicator Tray Containing E. coli and Antibiotics
Relative Fluorescence at 4 hrs.

	Antibiotic		
Concentration (ug/mL)	Ciprofloxacin	Cefuroxime	Cefoxitin
0.5	2537	7902	8181
1	2621	7983	8270
2	2461	7161	7120
4	2527	7598	3692
8	2424	6469	2974

As shown at all concentrations, the E. coli was sensitive to ciprofloxacin and low fluorescence counts were observed. The E. coli was resistant to the concentrations of cefuroxime and high fluorescence counts were observed. The E. coli was resistant to the 0.5, 1, and 2 ug/ml concentrations of cefoxitin and high counts were observed, but it was sensitive to the higher concentrations of cefoxitin and low counts were observed for 4 and 8 ug/ml. Thus, there is a correlation between the fluorescence and antibiotic concentration, demonstrating that the system of this invention can be used to assess the effects of antimicrobics and to determine the minimum effective concentration compositions.

EXAMPLE 9. Effect of Antibiotics on the Oxygen Consumption of E. coli with $Ru(BiPy)_3Cl_2$ Fluorescence Indicator

A 0.5 McFarland suspension of E. coli (ATCC #25922) in Broth A (see Example 1) was prepared using an A-Just nephelometer. The suspension was diluted to 1 x 10^7 CFU/ml in wells of an indicator tray prepared as in Example 5 ($Ru(BiPy)_3Cl_2$ indicator) containing the antibiotics ciprofloxacin, cefoxitin and cefuroxime at final concentrations of 0.5 to 8 ug/ml. The trays were incubated at 37°C for 4 hours and their fluorescence measured in a Fluoroskan II fluorometer. The results are listed in Table 5.

Table 5

Fluorescence from an Indicator Tray Containing
E. coli and Antibiotics
Relative Fluorescence at 4 hrs.

	Antibiotic		
Concentration (ug/mL)	Ciprofloxacin	Cefuroxime	Cefoxitin
0.5	507	1155	1171
1	428	1539	1491
2	308	1183	1338
4	403	1170	832
8	323	1194	559

As shown, as in Example 8, at these concentrations the E. coli is sensitive to ciprofloxacin and low fluorescence counts were observed. The E. coli is resistant to these concentrations of cefuroxime and high fluorescence counts were observed. The E. coli is resistant to the 0.5, 1, and 2 ug/ml concentrations of cefoxitin, high counts were observed; it was sensitive to higher concentrations of cefoxitin and lower counts were observed for 4 and 8 ug/ml. Thus, the results indicated that $Ru(BiPy)_3Cl_2$ can also be used in a fluorescence indicator.

EXAMPLE 10. Effect of Antibiotics on the Oxygen Consumption of Microorganisms Using DPA Fluorescence Indicator

A 0.5 McFarland suspension of E. coli (ATCC #25922) in Broth A was prepared using an A-Just nephelometer. The suspension was diluted to 1 x 10^7 CFU/ml in wells of an indicator tray prepared as in Example 5 (DPA indicator) containing the antibiotics ciprofloxacin, cefoxitin and cefuroxime at final concentrations of 0.5 to 8 ug/ml. The trays were incubated at 37°C for 4 hours and their fluorescence measured in a Fluoroskan II. The results are presented in Table 6.

Table 6

Fluorescence from an Indicator Tray Containing
E. coli and Antibiotics
Relative Fluorescence at 4 hrs.

	Antibiotic		
Concentration (ug/mL)	Ciprofloxacin	Cefuroxime	Cefoxitin
0.5	91	183	192
1	109	197	173
2	94	195	164
4	74	160	101
8	68	161	95

As shown, at these concentrations the E. coli is sensitive to ciprofloxacin and low fluorescence counts were observed. The E. coli is resistant to these concentrations of cefuroxime and high fluorescence counts were observed. The E. coli is resistant to the 0.5, 1, 2 ug/ml concentration of cefoxitin, high counts were observed; it was sensitive to higher concentrations and lower counts were observed for 4 and 8 ug/ml as in Examples 8 and 9, indicating that DPA is also useful as a fluorescence indicator.

EXAMPLE 11. Effect of Open and Closed Systems on Oxygen Measurements

A 96 well indicator microtiter tray was produced substantially as in Example 1. Duplicate wells in the tray were supplemented with the antibiotic cefuroxime in the concentration range of 0.25 to 32 ug/ml. One hundred and fifty microliters of a suspension of E. coli (ATCC #11775) was added to the wells to yield about 3×10^7 CFU/ml. One of each duplicate well was overlaid with mineral oil to inhibit diffusion of oxygen into the wells, the other duplicate was left open to the air. The tray was incubated at 37°C for 5 hours, the fluorescence was measured in a Fluoroskan II fluorometer and that fluorescence was compared with the average of several wells containing no antibiotic to yield a percent of the growth control at each antibiotic concentration. Figure 5A shows the behavior of the open and covered wells at five

hours as a function of cefuroxime concentration. Figure 5B shows the change in fluorescence of the growth control wells when open or overlaid with mineral oil.

The "closed system" overlaid with mineral oil did not show an effect on oxygen consumption by the 4 and 8 ug/ml concentrations of antibiotic while those wells with no mineral oil showed correctly that this organism is sensitive to cefuroxime at these concentrations. This difference is due, presumably to the time lag needed for the antibiotic to affect the organism; it is believed that during this time the oxygen is brought to an artificially low level by the ongoing metabolic activity of the organisms.

Thus, to utilize the invention with optimum sensitivity for the detection of the effect of toxins on organisms, the sample reservoir permits the influx of oxygen.

EXAMPLE 12. The Effect of Sample Volume on Indicator Trays

A 0.5 McFarland suspension of E. coli (BDMS Culture collection #7133) was diluted to 1 x 10^7 CFU/ml in Broth A. Different volumes (from 10 ul to 300 ul) of the diluted suspension were placed into wells of an indicator tray produced as in Example 1. The tray was incubated at 37°C and the fluorescence measured in a Fluoroskan II at 30 minute intervals. Fluorescence from the same volume of sterile broth was subtracted to give the fluorescence change cause by the microorganism. The results are presented in Table 7.

Table 7

Effect of Sample Volume on Indicator Tray Fluorescence

	Relative Fluorescence						
Sample Volume (ul)	0 hr.	1 hr.	2 hr.	2.5 hr.	3 hr.	3.5 hr.	4 hr.
10	0	0	0	0	0	4	139
20	0	0	275	795	814	1218	1958
40	0	245	683	1883	2108	2613	3240
60	0	80	1559	3497	4847	6226	6827
80	0	82	1798	5340	8333	8810	8801
100	0	31	1848	5952	7672	7962	7961
125	0	103	2798	6286	7580	7852	7852
150	0	32	2539	6005	6568	6759	6886
175	0	51	2574	6149	6993	6987	6798
200	0	59	2376	5355	5742	5944	5826
250	0	115	2172	5373	5695	5822	5759
300	0	107	2538	4650	4727	4825	4778

Briefly, it was observed that those wells with 40 ul or less of sample showed less that ½ the increase in relative signal observed in wells with 80 ul or more at times of 2 hours or more. It is believed that in the wells containing 40 ul or less, too little volume was present for the organisms to effectively consume oxygen faster than it could diffuse into the small volumes of sample.

EXAMPLE 13. Use of Indicator System Without a Fluorometer

Indicator trays were prepared using the same fluorescent compound and silicone as in Example 1. However, the trays were made of clear plastic and the wells had round bottoms (#4-3918-2, BD Labware, Lincoln Park, NJ). Two nanograms of $Ru(DPP)_3Cl_2$ in 10 ul of silicone were placed in each well of the tray and no wash steps were performed. Samples of Ps. aeruginosa (BDMS Culture

collection #N111) and E. coli (ATCC #25922) were diluted to Broth A (see Example 1) 1 x 10^7 CFU/ml in Broth A containing either 0 to 32 ug/ml cefuroxime, 0.12 to 8 ug/ml ciprofloxacin or 0 to 32 ug/ml cefoxitin and charged to the trays. The trays were incubated for 4 hours at 37°C and subsequently placed on the stage of an ultraviolet transilluminator (#TX-365A, Spectronics Corp., Westbury, NY) which served as an excitation source. The resulting fluorescence was observed from directly above the trays at a distance of 1 foot through a 550 cut-on filter (#LL-550-S-K962, Corion, Holliston, MA). It was readily observed that wells which contained either no antibiotics or concentrations of antibiotics that did not affect the organisms demonstrated a high level of fluorescence. Wells with either no organisms or higher antibiotic levels had a much lower level of fluorescence. The lowest concentration of antibiotic to significantly lower the fluorescent emissions for each organism is shown in Table 8 along with the MIC concentration determined using an overnight microdilution antimicrobial susceptibility test.

TABLE 8
Fluorescence Results Obtained Without Use of an Instrument
MIC

	Cefuroxime		Ciprofloxacin		Cefoxitin	
	Visual	Reference	Visual	Reference	Visual	Reference
Ps. aeruginosa	>64	>64	1	0.5	>64	>64
E. coli #25922	16	8	<0.12	<0.12	8	4

EXAMPLE 14. Use of Indicator to Detect the Presence of a Low Level of Bacteria In a Medium Containing Blood

Tissue culture flasks (Falcon #3084, BD Labware, Lincoln Park NJ) were prepared with one side coated with 3 mls of Dow Corning Water-based Emulsion containing 68 ng of $Ru(DPP)_3Cl_2$. The flasks were sterilized using ethylene oxide. One hundred thirty five mls of TSB broth (BBL #11768) containing about 0.05 CFU/ml E. coli (ATCC #25922) and 15 mls of defibrinated sheep blood was added to one of the flasks. A control flask contained 135 mls of TSB and 15 mls of blood but

no organisms. The caps of the flasks were loosened to allow air circulation and the flasks were incubated at 37°C in an upright position. A fiber optic probe allowed the fluorescence from the flasks to be measured by a Perkin Elmer LS-5B spectrofluorometer located several feet from the incubator. The fluorometer measured the flasks at 485 nm excitation wavelength with a 10 nm slit width and a 550 nm cut-on emission filter. A strip chart was attached to the fluorometer and the fluorescence monitored continuously for 16 hours. At 7.5, 10.5 and 16 hours during the incubation period a 100 ul aliquot was removed from the test flask, diluted 1:100 in sterile TSB and 100 ul of the dilution was spread on each of three TSA plates to determine the number of CFU/ml present in the flask. The results are graphically depicted in Figure 6.

As shown, the non-invasive techniques of this invention can be used for the detection of organisms in blood, a very critical and demanding task. The flask contained a very cloudy and turbid solution which is continuously monitored for sixteen hours, and measurement of fluorescence showed a direct correlation to the growth of organisms. This growth was readily detected by 11 hours, when the concentration of organisms had just exceeded 10^6 CFU/ml.

EXAMPLE 15. Indicator Coated on the Spherical Ends of FAST Tray Lid Prongs

This example monitored bacterial respiration with oxygen indicators coated on the spherical ends of FAST tray (Becton Dickinson) lid prongs. Three different indicators were evaluated.

The first indicator prepared was a mixture of 1 ml of 2 mg/ml dichloromethane solution of $Ru(DPP)_3Cl_2$ and 10 ml Dow-Corning 3-5024 water-based silicone emulsion. The spherical ends of FAST tray lid prongs were dipped into a shallow reservoir of the indicator solution, removed, placed prong side down in a rack, and allowed to cure by evaporation. The second indicator was prepared by mixing 3mL

Wacker SWS-960 clear silicone dispersion, 6 mL petroleum ether, and 0.5 mL of the 2mg/mL dichloromethane solution of $Ru(DPP)_3Cl_2$. The spherical ends of FAST tray lid prongs were coated with this indicator in the same manner as with the first indicator and allowed to cure by evaporation of the solvents and reaction with atmospheric moisture. The third indicator was prepared in the same manner as the second but Wacker SWS-960 white silicone was used.

A 1 x 10^7 CFU/mL suspension of E. coli ATCC #25922 in Mueller Hinton broth was prepared; 150 microliter aliquots were pipetted into the odd numbered rows of a microtiter tray, while 150 microliter aliquots of uninoculated Mueller Hinton broth were pipetted into the wells of the even numbered columns. The lids containing the indicator coated prongs were placed on the trays. The lidded trays were placed in a 37°C high humidity incubator for 3 hours.

Following the three hour incubation, the trays were placed on a transparent glass plate. A mirror was positioned below the glass plate in such a manner that the bottom of the tray was visible in the mirror. A 365 nm ultraviolet source which evenly illuminated the entire tray was positioned about one inch from the top of the tray. A box, with a small window through which the mirror could be seen, was placed over the assembly to block room light, and a 550 nm cut-on filter was placed in the box window. With this assembly the fluorescence from the indicator coated spherical ends of the FAST tray lid prongs could be visualized through the tray bottom. Table 9 contains the results of visual observations of the trays evaluated in this manner.

Table 9

Visual Observations of Indicator Coated Lid Prongs
Viewed Through Tray Bottoms

Silicone	Observations
Dow-Corning	Very bright fluorescence from spheres immersed in organism containing wells. Very weak fluorescence from prongs in uninoculated wells.
Wacker Clear	Some visible difference between prongs immersed in inoculated and uninoculated wells. Difference much less observable than with Dow-Corning indicator.
Wacker White	Very bright fluorescence from spheres immersed in inoculated wells, intensity about equal to Dow-Corning indicator. Some weak fluorescence from spheres in uninoculated wells.

Thus, all three indicator systems produced desirable results, with the Dow Corning and Wacker White exhibiting much more distinguishable differences between the inoculated and uninoculated wells.

EXAMPLE 16. Indicators Consisting of $Ru(DPP)_3Cl_2$ Adsorbed on Silica Gel Particles Embedded in UV Cured Silicone Rubber

Indicators were prepared by adsorbing $Ru(DPP)_3Cl_2$ onto silica gel particles and embedding these particles into Loctite Nuva-Sil silicone rubbers. A variety of indicators were prepared using silica gel particles of different mesh sizes, different amounts of adsorbed fluorophore, different ratios of silica gel to silicone, and two types of Loctite Nuva-Sil (Nuva-Sil 5091 and Nuva-Sil 5147). Table 10 contains the characteristics of the indicators prepared and the visual results obtained from the indicators in contact with microorganism suspensions. An exemplary procedure used for the preparation of the indicators is presented below.

Ten grams of 100-200 mesh Davisil silica gel (Aldrich, Milwaukee, WI) was weighed into a 500 mL round bottom evaporation flask. Forty three milliliters of a 0.14 mg/mL ethanol solution of $Ru(DPP)_3Cl_2$ was pipetted into the flask. The ethanol

was removed by rotary vacuum evaporation resulting in the adsorption of the $Ru(DPP)_3Cl_2$ on the silica gel at a concentration of 0.6 mg $Ru(DPP)_3Cl_2$/gm silica gel. Four grams of this silica gel were mixed with 16 g Loctiote Nuva-Sil 5091 (Locite, Newington, CT) resulting in a 20% w/w silica/silicone ratio. Twenty-five microliter aliquots of this mixture were pipetted into the wells of a microtiter tray. The silicone was cured by exposure to high intensity ultraviolet radiation for 15 seconds in a Loctite Zeta 7200 UV curing chamber. The other indicators in Table 10 were similarly prepared.

To evaluate the indicators, 150 microliters of a 1 x 10^7 CFU/mL suspension of E. coli (ATCC #25922 in Mueller Hinton II broth (BBL) was pipetted into selected wells of the microtiter tray; uninoculated broth was pipetted into other wells. The tray was incubated in a high humidity 35°C incubator for 3 hours. To visualize the fluorescence from the indicator the tray was placed on the stage of a 365 nm UV transilluminator; the fluorescence from the indicator was observed from above through a 550 nm cut-on filter. A "+" sign in the Response column of Table 10 indicates that a visibly discernible increased fluorescence was observed from the wells containing the organism.

Table 10

Indicator Formulations and Responses

Mesh Size	mg $Ru(DPP)_3Cl_2$/g Silica	Wt % Silica	Silicone	Response
60-100	0.2,0.4,0.6	5,10,20	5091,5147	+*
100-200	0.2,0.4,0.6	5,10,20	5091,5147	+*
200-425	0.2,0.4,0.6	5,10,20	5091,5147	+*

* Represents result from all 18 trials (9 each for Silicone 5091 and 5147).

In replicate trials utilizing wells with no microorganisms, the indicators displayed little or no light (although at higher (0.6 mg/gm) concentrations of indicator, a dim fluorescence was noted).

EXAMPLE 17. Oxygen S nsor Not In Direct Contact With Sample Fluid

Test vials (80 mL volume) containing 60 mL of media and oxygen sensor (OS) were inoculated with the following organisms: *Pseudomonas aeruginosa, Mycobacterium fortuitum* and *Escherichia coli.* The vials were connected to 80 mL vials without broth with oxygen impermeable rubber tubing. The vials were then entered into adjacent stations in a BACTEC® 9240 instrument. Data was collected on the two vials over a 50 hour period. The results of these tests are presented in Figures 7 through 9. Figure 7 depicts the data collected in the BACTEC® instrument indicating the change in fluorescence intensity indicative of the growth of *P. aeruginosa.* Figure 8 depicts the data collected in the BACTEC® instrument indicating the change in fluorescence intensity indicative of the growth of *M. fortuitum.* Figure 9 depicts the data collected in the BACTEC® instrument indicating the change in fluorescence intensity indicative of the growth of *E. coli.* For each of the figures the bold line in these figures represent the data collected in the vials containing broth; the light lines represent the data collected by the sensor that is not in direct contact with the liquid broth. In all three cases, oxygen consumption was observed in the vials without broth. The pattern of oxygen consumption exhibited in these vials indicates logarithmic oxygen consumption which is indicative of microbial growth.

The data shows that the OS was used for the detection of microbial growth in the absence of direct broth to sensor contact. The detection delays observed in the vials without media are related to this particular test configuration. One having ordinary skill in this art would be able to optimize the parameters of the system, by example and not limitation, such as, by reducing the headspace volume and oxygen concentration which would result in improved sensitivity and make the measurements made without direct contact of liquid broth (gas phase) more comparable with the measurements made with contact of the liquid broth (liquid phase).

EXAMPLES 18 - 26

Methods and Materials: Oxygen Sensor Plate preparation

Oxygen sensor plates were prepared by the general methods described herein. Falcon 1177 polystyrene 96 well U-bottom plates (BD Labware) were used for all experiments. The fluorescent dye $Ru(DPP)_3Cl_2$ was adsorbed to silica gel by rotary evaporation of ethanolic solutions of the dye with the silica gel. The adsorbed dye-silica and a moisture-cure clear silicone were mixed manually and immediately applied to plate wells with approximately 17 uL silicone per well. These were cured for 2-3 days in a controlled humidity incubator.

Microwell Plate Fluorescence Assays

All data was obtained with a BMG Polarstar fluorimeter at 37°C using the bottom plate reading configuration. The bandpass filters were 465 nm for excitation and 590 nm for emission. For the experiments with MATRIGEL®, a Cytofluor 4000 fluorometer was used with a 485 nm excitation filter and a 580 nm emission filter. Data was read at selected time intervals. Normalized fluorescence data was generally obtained by dividing well values at selected time points by the same well's initial reading with only media or buffer present prior to adding cells.

Cells used for cytotoxicity and quantitation experiments (HL60, U937; ATCC) were grown in tissue culture media recommended by the supplier (RPMI; Gibco and ATCC, respectively). The media was supplemented with either 20% fetal bovine serum (Hyclone) for the HL60 cells or 10% FBS for the U937 cells, with the addition of penicillin, streptomycin and fungizone (Gibco) to prevent microbial contamination. Cells were maintained in a tissue culture incubator (37°C, 5% CO_2, 95% humidity) during all experiments between readings.

EXAMPLE 18. Quantitation of Fluoresc nt Signal vs. Cell Number for HL60 Cells Growing in Oxygen S nsor Plates

HL60 cells (human promyelocytic leukemia cell line, ATCC #45500) were grown in tissue culture media recommended by the supplier (RPMI; Gibco), supplemented with 20% fetal bovine serum, heat inactivated at 50°C for thirty minutes with the addition of penicillin, streptomycin and fungizone (Gibco) to prevent microbial contamination. Cells were maintained in tissue culture incubator (37°C, 5% CO_2, 95% humidity) during all experiments. Fluorescence of the oxygen sensor was read on a Polarstar™ fluorometer (BMG), using 465 nm excitation and 590 nm emission filters.

100 uL of tissue culture media was aliquoted into the wells of the Oxygen sensor plate and plate was allowed to equilibrate in the tissue culture incubator for 1 hour prior to taking the initial reading. This reading was used to normalize all subsequent readings to account for well to well variability. Cells were resuspended in fresh media at 960,000 cells/ml. Serial 1:2 dilution of this stock was performed and 100 uL of each dilution was alliquoted across the length of the plate in replicates of 12 (rows B-H), with final cell/well number from 1,500 to 96,000. In row A, 100 uL of tissue culture media was alliquoted into the wells in lieu of cells (no-cells control). Fluorescence measurements were taken every 24 hours over 5 days. The actual number of cells present was counted with a hemocytometer by sampling the parallel well (same seeding cell number). Mean fluorescence from 5 wells per data point (n=5) was plotted against the counted cell number (Figure 10). Error bars are standard deviation of the mean.

EXAMPLE 19. Quantitation of Fluorescent Signal vs. Cell Number for U937 Cells Growing in Oxygen Sensor Plates

This experiment was performed similarly to the one in Example 18. U937 cells (human histiocytic lymphoma cell line, ATCC, #CRL-1593.2) were grown as above, with the exception that 10% fetal bovine serum was used. Cell number

varied from 750 to 48,000 cells per well. Fluorescence was measured at times indicated and plotted against seeded (initial) cell number (Figure 11).

EXAMPLE 20. Cytotoxicity of Vinblastine Assayed By Oxygen Sensor

The experiment was performed as described in Example 18, with the following exceptions. Serial dilutions of vinblastine were prepared at twice the final concentration (100 nM to 0.1 nM) in tissue culture media.100 uL of the drug dilution in tissue culture media was aliquoted across the width of the plate in replicates of five, reserving one column for no-drug (media only) control. After an initial reading, a constant number of cells (200,000/well) in 100 uL media were added to each well of rows A-E of the oxygen sensor plate, reserving rows F-H for no-cells control. Fluorescence was read at the indicated times. Mean fluorescence from 5 wells per data point (n=5) was plotted against vinblastine concentration (Figure 12).

EXAMPLE 21. Cytotoxicity of Vinblastine Assayed by MTT.

In parallel with the oxygen sensor assays, MTT assays were performed using a Cell Titer Kit™ (Promega), as in Example 20, with the following exceptions. For each time point (corresponding to oxygen sensor assay time point), one flat-bottom 96 well microtiter plate (Falcon) was used. 50 uL of drug dilution in tissue culture media was used for initial reading and cells were suspended in 50 uL media, to the final volume of 100 uL. At indicated time points, 10 uL of MTT reagent was added per well for 1 hour, after which 100 uL of the stop/lysis buffer was added. Plates were sealed with Parafilm™ and cell lysis occurred overnight. Absorbance (570nm corrected by absorbance at 750nm) was read using a Thermomax Microplate Reader (Molecular Devices). Mean absorbance from 5 wells per data point, with standard deviation as error bars, was plotted against vinblastine concentration (Fig. 13).

EXAMPLES 22-24 Cytotoxicity of Methotrexate, Sodium Azide, and SDS (Sodium Dodecyl Sulfat) Assayed by Oxygen Sensor Plates and MTT Assays

These experiments was performed as described in Example 20, with the exception that appropriate dilutions of the above reagents were substituted for vinblastine: 0.01 nM to 10000 nM methotrexate, 0.00001 to 10 mM sodium azide, and 2 to 2000 uM SDS respectively. Complete dose response curves for these three additional drugs are shown, respectively in Figures 14, 15 and 16.

Table 11: Comparison of IC_{50} Values For Selected Drugs With HL60 Cells Obtained With Oxygen Sensor Plates and MTT Assays**

Time (hours)	24		48		72	
	O_2 Sensor	MTT	O_2 Sensor	MTT	O_2 Sensor	MTT
Vinblastine	*	*	11 nM	9 nM	11 nM	8 nM
Methotrexate	*	*	*	*	*	*
SDS	340 uM	500 uM	300 uM	440 uM	300 uM	430 uM
Sodium Azide	*	*	13 uM	25 uM	13 uM	25 uM

Time (hours)	96		120	
	O_2 Sensor	MTT	O_2 Sensor	MTT
Vinblastine	9 nM	7 nM	9 nM	6.4 nM
Methotrexate	19 nM	*	12 nM	14 nM
SDS	*	*	*	*
Sodium Azide	9 uM	3 uM	5.8 uM	1.6 uM

* There was not a suitable sigmoidal dose response curve or that data were not sampled at this timepoint.

** 50% Inhibitory Concentration.

This corresponds to Oxygen Sensor Experiments described in Examples 20 and 22-24. MTT data was obtained by general methods described in Example 21. The IC_{50} values were determined as the concentration of drug that decreased the assay signal by 50%. IC_{50} values were determined by a four-parameter logistic curve.

Discussion (for Table 11 and Examples 20-24):Cytotoxicity assays were performed in parallel with oxygen sensor plates and with standard MTT assays to measure the cytotoxicity of four drugs/toxins: vinblastine, methotrexate, sodium azide, and sodium dodecylsulfate. Both assay methods gave comparable IC_{50} values at the selected

timepoints. Because the MTT assay is an endpoint assay requiring additional reagents and destruction of the cells, it required a separate plate for each timepoint. The oxygen sensor assay, however, required only a single plate for all timepoint readings with each drug, significantly reducing the amount of labor and materials for each IC_{50} determination over time.

Conclusion: For each timepoint in these cytotoxicity assays where significant drug or toxin effect could be measured, the IC_{50} values obtained for the oxygen sensor plate matched closely with the MTT values.

EXAMPLE 25. Oxygen Consumption by Mammalian Cells Growing on MATRIGEL®

Various amounts (100 µl, 50 µl, 25 µl, or 0 µl) of MATRIGEL® (BD Labware cat. # 4024C) were added to the wells of an ice-cold O_2 sensor tray and then allowed to gel at room temperature. The plate was moved to a 37°C incubator before use. MCD-1 cells (Moore et al. (1996) In Vitro Properties of a Newly Established Meulloblastoma Cell Line MCD-1. Mol. Chem. Neuropath. 29, 107-126) were suspended by trypsinization, washed with DMEM/F12 medium containing 10% fetal calf serum, and resuspended to 5 x 10^5 cells/ml in the same medium without serum. In some experiments, Hepes buffer (10 mM, pH 7.4) was added to better control the pH during the incubation period. Cells (100 µl, 5 x 10^4 cells) were added to the wells and the plate was moved to a modified PerSeptive Biosystems Cytofluor fluorimeter at 37°C. The fluorescence was read over time using 485 nm excitation and 580 nm emission filters.

There was a clear increase in fluorescence signal in wells containing MCD-1 cells compared to wells without cells (Figure 17). The signal increased more slowly in wells containing increasing amounts of MATRIGEL®, suggesting there may be a barrier to the migration of cells toward the silicone sensor or to the diffusion of

oxygen through the MATRIGEL®. Much of the increase in signal occurred over the first few hours of the experiment.

EXAMPLE 26. MCD-1, SK-N-SH, and NIH3T3 Cells Growing on MATRIGEL® Differ in Their Rates of Oxygen Consumption

The O_2 sensor with or without 50 µl MATRIGEL® per well was prepared as described above. MCD-1, SK-N-SH (American Tissue Type Collection HTB11), or mouse fibroblast NIH3T3 cells (American Tissue Type Collection CRL1658) were collected by trypsinization, washed and added to wells as above at 5×10^3 or 5×10^4 cells per well. The plates were moved to a 37°C fluorimeter and monitored as above.

For MCD-1 cells (Figure 18A), the fluorescence signal developed more slowly in the wells containing MATRIGEL® than in the wells lacking MATRIGEL®. However, the final levels of fluorescence were comparable. The signal with 5×10^3 MCD-cells per well was much smaller than the signal with 5×10^4 cells, but was above the "no cells" control. In addition, the oxygen consumption by the MCD-1 cells was inhibited by 0.1% sodium azide, as also observed for suspension cell cultures. The signal with 5×10^5 SK-N-SH cells (Figure 18B) was very clearly above background and moreover, appeared to show a second increase beginning at about 6 hr, perhaps due to cell division. A third cell line, 3T3, showed an oxygen consumption between that of MCD-1 and SK-N-SH cells (Figure 18C). Although the three cell lines differ markedly in their metabolic rates, oxygen consumption could be detected in all cases by a proper choice of cell number.

Table 12 presents a summary for some of the mammalian cell types and corresponding conditions for growth which have been used with oxygen sensor plates.

Table 12: Cell lines which have been tested in Oxygen Sensor Plates

Adherent Cells

Cell Line	Cell type	Media	Contains	Supplements
SK-N-SH	Human	S-MEM		Pen/Strep/Fungizone
	Neuroblastoma	Gibco	Eagles Salts	Nonessential Amino Acids
		cat # 11380	L-glutamine	Sodium Pyruvate
		Contains		10% Fetal Bovine Serum
MCD-1	Human	D-MEM F12	15 mM Hepes	Pen/Strep/Fungizone
	Medulloblastoma	Gibco	L-glutamine	10% Fetal Bovine Serum
		cat # 11330	pyrodoxine HCl	
WI-38	Human	MEM	Earles Salts	Pen/Strep/Fungizone
	Fibroblast	Gibco	L-glutamine	Nonessential Amino Acids
	Embroyonic lung	cat # 11095		Sodium Pyruvate
				10% Fetal Bovine Serum
NIH-3T3	Mouse	D-MEM F12	15 mM Hepes	No supplements
	Fibroblast	Gibco	L-glutamine	
		cat # 11330	pyrodoxine HCl	

Nonadherent Cells

Cell Line	Cell type	Media	Contains	Supplements
HL60	Human	RPMI	L-glutamine	Pen/Strep/2X Fungizone
	Promyelocytic	Gibco		20% Fetal Bovine Serum,
	Leukemia	cat # 11875		heat inactivated
U-937	Human	RPMI	L-glutamine	Pen/Strep/2X Fungizone
	Histiocytic			
	Lymphoma	Gibco		10% Fetal Bovine Serum
		cat # 11875		
		ATCC	10 mM Hepes	Pen/Strep/2X Fungizone
		cat # 30-2001	1 mM Na pyruvate	10% Fetal Bovine Serum
			4 g/L glucose	
			1.5 g/L bicarbonate	
			2 mM glutamine	

EXAMPLE 27. Oxygen Sensor Added to Bottom of Well Ins rt Membrane

This example demonstrates an alternate format for using the sensors to monitor cells and provides a method for monitoring the growth of an adherent cell line (MDCK) by applying the sensor to the exterior of a cell culture insert membrane which supports growth of adherent cells.

The oxygen sensor silicone formulation was prepared by the general methods described herein and 50 uL was applied to one half of the wells of a 24 well microplate (Falcon product 3047). In addition, 20 uL of the sensor silicone mixture was added to the exterior side of the track-etched PET (polyethylene terephthalate) membrane of 24-well plate inserts (Falcon 3097). These sensors were cured at 37°C for 2 days.

Cells in these modified inserts were monitored in the unmodified wells of the 24 well plate and compared to corresponding cell lines grown in modified wells with the sensor on the well bottom but without an insert present. Cells were grown and monitored by fluorescence as in the preceding examples. Briefly, 300,000 HL60 cells were added to each modified insert or well and 100,000 MDCK cells were added to each insert or well. The cells were added in the corresponding media for each cell type (see Table 12): 0.7 mL per well and 0.3 mL per insert. Control wells with media only and no cells were also monitored. Readings were taken daily for nine days.

The results are shown in Figures 19A and 19B. For both the adherent cells and non-adherent cells a greater signal was obtained more quickly with the modified insert sensors.

EXAMPLE 28. Preparation of a 384 Well Sensor Plate

The general methods described above and in Example 18 were used to prepare a 384 well sensor plate (Nunc #242765 clear polystyrene) with 10 uL of

sensor per well. A titration of HL60 cells in 100 uL media was monitored with the BMG fluorescence plate reader.

The results are shown in Figure 20 and compared to the corresponding 96 well plate results. The 384 well plate demonstrated an improved time-to-signal response for the same number of HL60 cells per well.

EXAMPLE 29. Detection of SF-9 Insect Cell Growth

SF-9 insect cells are derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda* (D. R. O'Reilly, et al (1992) *The Baculovirus System: A Laboratory Guide*, Chapman and Hall, NYC, NY). A 96 well oxygen sensor plate was equilibrated for 1 hour at 27 °C with 100 μL TMN-FH media (Invitrogen, Inc., Graces Insect media supplemented with 10 % fetal calf serum, and powdered form of yeastolate, lactalbumin hydrolysate and glutamine).

Seven concentrations of serially diluted SF-9 cells were added in replicates of five across the O_2 sensor plate starting at 160,000 cells/well (800,000 cells/mL) down to 1,500 cells/well (7,500 cells/mL). Cells were incubated at 27°C in a humidity chamber and fluorescence was monitored over time using the same instrument and parameters used in the mammalian cell experiments.

The signal increased more rapidly and to a greater degree than mammalian cells (Figure 21). The wells with 160,000 cells/well reached maximum fluorescence within 2 hours. As with other cellular experiments, the initial cell number in a well can be estimated by the time required to reach a measurable fluorescence increase per well.

EXAMPLE 30. Monitoring Yeast Growth

Growth of a sample of *Saccharomyces cerevisiae* was monitored in a 96 well oxygen sensor plate (prepared as described in Example 16). An initial yeast broth was prepared by hydrating 11.5 g of dried Edme Ale Yeast (Edme, Ltd., UK) in 100

mL water at 37 °C for 30 minutes. This yeast slurry was added to a 500 mL mixture of NZCYM Media (BBL #99165) plus 10 g/L d-glucose. After 36 hours of fermentation at 27 °C (just past exponential growth phase) the yeast cell concentration was determined using a hemacytometer. Serial dilutions were prepared from 1.2×10^7 cells/mL down to 1.6×10^4 cells/mL in fresh NZCYM broth with either 10, 50, 100, or 200 g/L d-glucose. Each suspension was measured in triplicate (200 uL /well) in a 96 well sensor plate at 27 °C with continuous readings every 400 sec for 13.3 hours.

Figure 22 shows the fluorescence signal for the yeast cell titration in 10 g/L glucose. This indicates the relationship between initial concentration and time required to generate a positive signal. Figure 23 compares yeast growth (as determined by time required to reach 120% of initial fluorescence) vs. glucose concentration. The two lower glucose concentrations (10 and 50 g/L) indicate a more linear relationship to the initial yeast concentration, whereas the two higher glucose concentrations (100 and 200 g/L) appear to retard initial growth of low yeast concentrations. This demonstrates one way the sensor plates can be used for optimizing cellular growth conditions.

It is apparent that many modifications and variations of this invention as hereinabove set forth may be made without departing from the spirit and scope of the present invention and the above examples are not intended to in any way limit the present invention but are merely exemplary.

EXAMPLE 31. Monitoring Enzymatic Activity of Glucose Oxidase

The oxidation of glucose by glucose oxidase was monitored in a 96-well oxygen sensor plate (prepared as described in Example 16). Glucose oxidase low in catalase content was purchased from Sigma and was diluted at the time of use in Delbecco's phosphate buffered saline (DPBS). A sterile solution of 2 M glucose (in DPBS) was likewise diluted at the time of use in DPBS. Reaction mixtures consisting

of 200 uL of glucose were added to wells and allowed to incubate at room temperature in the absence of enzyme for 30 minutes, at which time baseline fluorescent readings were taken. Enzyme (at a starting at a concentration of 500 U/mL) was subsequently added and the plates were read periodically over the course of an hour.

It can be seen from the results plotted in Figure 24 that a reaction system featuring 5 U/mL glucose oxidase gives rise to a measurable extent of fluorescence for glucose concentrations of 1 mM or greater. Increasing the concentration of enzyme had no significant impact on the ability to detect very low concentrations of glucose (data not shown). In all cases, the fluorescent signal achieved steady-state within approximately 30 minutes. Because this system is in equilibrium with atmospheric oxygen, the steady-state fluorescent signal corresponds to the steady-state concentration of oxygen in the well, which is in turn dependent upon the concentration of the enzyme and the glucose. Under the conditions of this test, it may be concluded that the rate of oxygen consumption for a reaction mixture containing a glucose concentration of 1 mM or lower is too low to overcome the rate of diffusion of oxygen in from the atmosphere. As a result, there is no measurable impact on the concentration of dissolved oxygen in the aqueous media and no increase in fluorescence.

Figure 25 demonstrates that the steady-state level of fluorescent signal resulting from such reactions is dependent upon the concentrations of both glucose and glucose oxidase. This steady-state reading corresponds directly to the steady-state level of dissolved oxygen in the media in a fashion consistent with the data shown in Example 1. At very large concentrations of enzyme and substrate, the signal reaches a maximum, indicating that the oxygen concentration has been reduced to zero, as would be expected for a reaction system with a very fast rate of oxygen consumption.

EXAMPLE 32. Bas line Enzymatic Activity of Microsomes

The cytochromes ("CYP") P450 are a large family of proteins, found predominantly in the liver, involved in the metabolism or detoxification of substances in the body such as chemicals, hormones, or natural food substances. While there are many different sub-families of CYP450, the isozyme families primarily involved in metabolism of xenobiotics are the CYPs 3A, 2C, 2D and 1A.

In order to assess the baseline ("uncoupled") level of oxidation exhibited by CYP450-containing microsomes in the absence of substrate, a set of experiments was conducted in a 96-well oxygen sensor plate (prepared as described in Example 16). Microsomes prepared from human lymphoblasts transfected with human CYP-2C9 were purchased from Gentest. Reaction buffer was prepared according to Gentest (100mM Tris) to which was added 3.3mM $MgCl_2$ and a regenerating system: 1mM $NADP^+$, 3.3mM Glucose-6-phopshate and 0.4units/ml glucose-6-phosphate dehydrogenase (Sigma). Reactions were also run under non-regenerating conditions by the addition of 1mM NADPH (Sigma) alone. Reaction buffer (195ul/well) was plated on the 96-well oxygen sensor plate and equilibrated at 37°C for 30 minutes. Reaction was initiated by the addition of 10 pmol/well CYP-2C9 and were monitored for consumption of oxygen over time.

Figure 26 shows a rise in the fluorescence signal with increasing amounts of CYP-2C9 (5 pmol, 10 pmol and 12 pmol). This indicates that the background level of oxygen consumption for the P450s is dependent on the concentration of the P450, as would be expected.

EXAMPLE 33. Oxidative Activity of CYP-2C9 Microsomes in Presence of Diclofenac

To demonstrate that the addition of a xenobiotic known to be metabolized by the P450 in question increases the level of oxygen consumption over the background level of the uncoupled reactions, additional experiments were conducted

as described above for Example 32, with the following modifications. Diclofenac, a known substrate of CYP-2C9, was added to the reaction mixture of certain wells prior to plating such that its final concentration was 200 uM. Diclofenac was prepared in DMSO (final concentration 0.04%); control reactions with P450 alone were treated with 0.04% DMSO. It can be seen that DMSO had a slight inhibitory effect on the level of oxygen consumption. Figure 27 demonstrates that the addition of 200 uM diclofenac resulted in an increase in oxygen consumption, indicative of an overall increase in metabolic activity as a preferred substrate was introduced into the system.

EXAMPLE 34. Enhancing Signal Due to Oxidative Activity of CYP-2C9 Microsomes in Presence of Diclofenac

Experiments were conducted as in Example 32, with the following variation. Some wells had 50 uL of mineral oil added on top of the aqueous media to minimize the rate of oxygen diffusion into the media from the atmosphere. As can be seen in Fig. 28, this modification in protocol increases the discrimination between the signal in the presence of substrate to that of the baseline.

EXAMPLE 35. Inhibiting Oxidative Activity of CYP-2C9 Microsomes in Presence of Diclofenac

To demonstrate that the increase in oxygen consumption was attributable to the oxidation of the drug, a known CYP-2C9 inhibitor (sulphaphenazole) was added to certain wells. Figure 29 shows that the observed increase in oxygen consumption due to the oxidation of diclofenac (500 uM) was specifically inhibited by the addition of sulphaphenazole (250 uM).

EXAMPLE 36. Oxidative Activity of CYP-2C9 Microsomes in Presence of Ibuprofen

To demonstrate the ability to measure oxidative metabolic reactions for other systems, the metabolism of ibuprofen, a drug known to have a moderate rate of

clearance by CYP-2C9, was measured using the oxygen sensor plate. Experiments were conducted as described in Example 32, with the following modifications. Ibuprofen was added to certain wells at a final concentration of either 100 or 500 uM. In addition, the amount of hydrogen peroxide formed was measured after termination of the reaction,by addition of Amplex Red™ (Molecular Probes), a fluorescent indicator of H_2O_2 levels.

As seen in Figure 30, increasing amounts of ibuprofen (100 uM and 500 uM) effectcorresponding increases in oxygen consumption over that of CYP-2C9 (10 pmol/well) alone. That the increase in oxygen consumption relates to the oxidation of ibuprofen is corroborated by the fact that the production of H_2O_2 (Figure 31) decreases with increasing amounts of drug (500 uM ibuprofen). These results together imply that the overall rate of oxidative activity is increased and that the extent of uncoupled activity has decreased in the presence of 500uM Ibuprofen.

EXAMPLE 37. Oxidative Activity of CYP-2D6 Microsomes in Presence of Propranolol

A second P450 enzyme system (CYP- 2D6) was used to demonstrate that the ability to measure the increase in oxygen consumption effected by drug metabolism transcends a single enzyme system. Here, reactions were conducted as described in Example 32a, except that a CYP-2D6 microsome system was used in a buffer system of 100mM Potassium Phosphate, pH 7.4, and the drug was propranolol, at final concentrations of 10 and 50 uM. Figure 32b demonstrates that the addition of propranolol to CYP-2D6 increased the consumption of oxygen relative to the no-drug control. That the increase in oxygen consumption above CYP-2D6 alone resulted from the oxidation of propranolol is corroborated by the fact that there was no change in the amount of H_2O_2 produced from the uncoupled reactions (data not shownIn addition, the consumption of NADPH, a co-factor for all three p450 reaction pathways, was measured concurrently with oxygen consumption, using the natural fluorescence of the molecule.

These experiments revealed that there was a slight increase in NADPH consumption in the presence of propranolol (relative to CYP-2D6 alone) that is consistent with the observed increase in oxygen consumption. These results together imply that the overall rate of oxidative activity is increased in the presence of propranolol, even though the extent of uncoupled activity has remained unchanged, and hence, that a small fraction of the total oxidative activity is due to the productive pathway. The ability to deduce this is dependent upon the ability in the present invention to be able to make substantially simultaneous measurements of oxygen and NADPH consumption, as well as H_2O_2 production.

EXAMPLE 38. Comparing Oxidative Activity of CYP-2D6 Microsomes in Presence of Propranolol and Dextromethorhpan

To demonstrate that different substrates effect different rates of oxygen consumption, a side-by-side experiment was conducted according to the protocol of Example 37, with the following modification: some wells received 100 uM propranolol and some received 100 uM dextromethorphan. As can be seen in Fig. 33a, a greater rate of oxygen consumption was observed for the wells containing propranolol which is known to have a higher rate of metabolism relative to dextromethorphan. In these experiments, the increased consumption of NADPH in the metabolism of propranolol relative to that of dextromethorphan is consistent with the greater oxygen consumption seen for propranolol due to its oxidative metabolism (Figure 33b).

EXAMPLE 39. Monitoring Oxidative Metabolic Activity in Intact Hepatocytes

The liver is the primary tissue for xenobiotic detoxification and oxidation such that the compounds can be eliminated from the body. This is accomplished through families of detoxification enzymes, including the P450s described above, as well as a number of other enzyme systems. These enzyme families reside in liver-specific cells called hepatocytes. Unlike the defined system of P450s described above, intact

hepatocytes contain all the different enzyme systems that could be contributing to the metabolic process. Total metabolic analysis can be performed by using *in vivo* models, liver slices or by isolation of the hepatocytes.

These studies employed "Liverbeads" (Biopredic) which are primary rat hepatocytes embedded within beads of alginate. There are approximately 300 hepatocytes per bead. Liverbeads were rapidly thawed in 15mls of L15 Leibovitz medium with 0.6 M D-glucose, mixed and rinsed 2x with 15mls of Williams E medium (2 mM Glutamate, 4ug/ml Bovine Insulin and 10% v/v fetal calf serum). The liverbeads were resuspended into Williams E medium (2 mM Glutamate, 4 ug/ml Bovine Insulin and $5x10^{-5}$ M Hydrocortisone hemisuccinate) and plated onto the Oxygen Biosensor 96-well platform. Liverbeads were plated at increasing density: 90, 60 and 45 Liverbeads which corresponded to 27,000, 18,000 and 14,000 hepatocytes per well. Liverbeads were incubated at 37°C with 5% CO_2 for a 4 hour time course.

As shown in Figure 34, increasing number of Liverbeads corresponded to an increase in the level of oxygen consumption for the 4 hour period measured, as would be expected. This signal is due simply to the baseline respiration of these cells, and that the signal correlates to the number of cells is consistent with expectation.

To determine whether the oxygen biosensor could be used in conjunction with hepatocytes to assess xenobiotic metabolism, the consumption of oxygen by the Liverbeads was measured in presence of diclofenac. 45 Liverbeads (~14,000 hepatocytes) were plated onto the oxygen biosensor as described above. Cells were incubated at 37°C for 30 minutes, and then 200 uM diclofenac (in 0.01% EtOH) was added to the cells. Readings for the oxygen biosensor were taken every 30 minutes for 4 hours. As shown in Figure 35, a significant increase in oxygen consumption occurred in the presence of diclofenac, relative to the Liverbeads alone. These data

demonstrate that even in a complex system such as a hepatocyte, the oxidation of a xenobiotic could be measured using the consumption of oxygen as one marker for compound clearance.

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